invitrogen™

BLOCK-iT™ Pol II miR Validated miRNA Control Vectors

Gateway®-adapted expression vectors containing validated microRNA (miRNA) sequences for use as positive controls

Catalog nos. V49350-00, V49351-00, V49352-00

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User Manual

Table of Contents

Table of Contents iii
Kit Contents and Storage iv
Accessory Products v

Introduction 1

Overview 1
Validated miRNA Control Vectors 5
Using miRNA for RNAi Analysis 8
Green Fluorescent Protein 12

Methods 14

Using the Validated miRNA Control Vectors 14
Transfecting Cells 16
Detecting Fluorescence 19
Generating a Stable Cell Line 20
Transferring the Pre-miRNA Expression Cassette to Destination Vectors 22
Troubleshooting 25

Appendix 27

Recipes 27
Blasticidin 28
Map and Features of pcDNA6.2™-GW/EmGFP-miR Validated miRNA Control Vector 29
Map of pcDNA™6.2-GW/EmGFP-miR-neg Control Plasmid 31
Technical Service 32
Purchaser Notification 33
Gateway® Clone Distribution Policy 36
References 37

Kit Contents and Storage

Types of Products

This manual is supplied with the following products:

Product	Catalog no.
BLOCK-iT™ Pol II miR-*lacZ* Validated miRNA Control Vector	V49350-00
BLOCK-iT™ Pol II miR-*luc* Validated miRNA Control Vector	V49351-00
BLOCK-iT™ Pol II miR-LMNA Validated miRNA Control Vector	V49352-00

Shipping and Storage

The BLOCK-iT™ Pol II miR Validated miRNA Control Vectors are shipped on dry ice. Upon receipt, store the vectors at -20ºC. Product is guaranteed stable for six months from date of shipment when stored properly.

Contents

The contents of each BLOCK-iT™ Pol II miR Validated miRNA Control Vector are described below.

Store the vectors at -20°C.

Item	Composition	Amount
BLOCK-iT™ Pol II miR-*lacZ* Validated miRNA Control Vector		
pcDNA™6.2-GW/EmGFP-miR-*lacZ* Validated miRNA Control Vector	0.5 µg/µl in TE Buffer, pH 8.0	20 µl
pcDNA™6.2-GW/EmGFP-miR-neg Control Plasmid	0.5 µg/µl in TE Buffer, pH 8.0	20 µl
BLOCK-iT™ Pol II miR-*luc* Validated miRNA Control Vector		
pcDNA™6.2-GW/EmGFP-miR-*luc* Validated miRNA Control Vector	0.5 µg/µl in TE Buffer, pH 8.0	20 µl
pcDNA™6.2-GW/EmGFP-miR-neg Control Plasmid	0.5 µg/µl in TE Buffer, pH 8.0	20 µl
BLOCK-iT™ Pol II miR-LMNA Validated miRNA Control Vector		
pcDNA™6.2-GW/EmGFP-miR-LMNA Validated miRNA Control Vector	0.5 µg/µl in TE Buffer, pH 8.0	20 µl
pcDNA™6.2-GW/EmGFP-miR-neg Control Plasmid	0.5 µg/µl in TE Buffer, pH 8.0	20 µl

Product Qualification

The structure of each vector is verified by restriction enzyme digestion. In addition, the pre-miRNA insert in each BLOCK-iT™ Pol II miR Validated miRNA Control Vector is sequence verified using the appropriate sequencing primers. The pre-miRNA insert must be 100% identical to the provided sequence (see page 11 for the insert sequence).

Accessory Products

Additional Products

The following reagents maybe used with the BLOCK-iT™ Pol II miR Validated miRNA Control Vectors. Ordering information for the products is provided below.

Product	Amount	Catalog no.
BLOCK-iT™ Pol II miR Validated miRNA Vector DuoPak	1 kit	V49300-XX*
BLOCK-iT™ Pol II miR RNAi Expression Vector Kit	20 reactions	K4935-00
BLOCK-iT™ Pol II miR RNAi Expression Vector Kit with EmGFP	20 reactions	K4936-00
BLOCK-iT™ RNAi Target Screening System (w/*lacZ* reporter)	20 reactions	K4916-00
Lipofectamine™ 2000 Reagent	0.75 ml 1.5 ml	11668-027 11668-019
Opti-MEM® I Reduced Serum Medium	100 ml 500 ml	31985-062 31985-070
Blasticidin	50 mg	R210-01
Ampicillin	200 mg	11593-019
Luria Broth Base (Millers LB Broth Base)	500 g	12795-027
Gateway® LR Clonase™ II Enzyme Mix	20 reactions 100 reactions	11791-020 11791-100
Gateway® BP Clonase™ II Enzyme Mix	20 reactions 100 reactions	11789-020 11789-100
One Shot® TOP10 Chemically Competent *E. coli*	20 x 50 µl	C4040-03
One Shot® Stbl3™ Chemically Competent *E. coli*	20 x 50 µl	C7373-03
pDONR™221	6 µg	12536-017
PureLink™ HiPure Plasmid Midiprep Kit	25 preps	K2100-04
PureLink™ HQ Mini Plasmid Purification Kit	100 preps	K2100-01
T4 DNA Ligase	100 units 500 units	15224-017 15224-025

*Visit www.invitrogen.com/RNAiExpress for details on the BLOCK-iT™ Pol II miR Validated miRNA Vector DuoPaks that are available from Invitrogen.

Spectinomycin

For selection of pcDNA™6.2-GW/EmGFP-miR transformants in *E. coli*, you will need to purchase spectinomycin, if you are using spectinomycin as the selection agent. Spectinomycin dihydrochloride is available from Sigma (Catalog no. S4014). For a recipe to prepare spectinomycin for use, see page 27.

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Accessory Products, Continued

Gateway® Destination Vectors

A large selection of Gateway® destination vectors are available from Invitrogen to facilitate the transfer of the pre-miRNA sequence into a suitable destination vector to allow the miRNA expression in multiple systems including viral expression and tissue-specific expression. See page 23 for a list of destination vectors compatible with the pcDNA™6.2-GW/EmGFP-miR Validated miRNA Control Vector.

BLOCK-iT™ RNAi Products

A large variety of BLOCK-iT™ RNAi products are available from Invitrogen to facilitate RNAi analysis including Stealth™ RNAi, the Validated Stealth™ RNAi Collection, and a large selection of RNAi vectors.

For details, visit the RNAi Central portal at www.invitrogen.com/rnai or contact Technical Service (see page 32).

Introduction

Overview

Introduction

Each BLOCK-iT™ Pol II miR Validated miRNA Control Vector (referred to as Validated miRNA Control Vector) includes a highly effective, functionally tested pcDNA™6.2-GW/EmGFP-miR Validated MiRNA Control plasmid containing a 68 bp insert which encodes a small hairpin precursor microRNA (pre-miRNA) molecule designed to target the mRNA of the specified gene.

The Validated miRNA Control Vectors are designed for *in vitro* and *in vivo* RNA interference (RNAi) analysis of a specific target gene.

Validated miRNA Control Vectors

The BLOCK-iT™ Pol II miR Validated miRNA Control Vectors are supplied in a ready-to-use format and target a specified region of the gene of interest, demonstrating at least 70% target gene knockdown as measured in a cell-based assay using reporter constructs or the pSCREEN-iT™/*lacZ*-DEST screening vector and components of the BLOCK-iT™ RNAi Target Screening System available from Invitrogen (see page v).

The Validated miRNA Control Vectors combine the BLOCK-iT™ Pol II miR RNAi Technology (see page 3) and Gateway® Technology (see page 4) to provide a fast and efficient method to perform RNAi analysis.

The Validated miRNA Control Vectors are Gateway®-adapted expression clones containing the pre-miRNA sequence of interest designed for transient or stable expression of the miRNA of interest in mammalian systems.

For details on how the Validated miRNA Control Vectors were generated, see page 5.

Applications

The Validated miRNA Control Vectors are ideal for use with your mammalian cells to familiarize yourself with the vector based miRNA expression system, if you are a first time user of this technology.

The Validated miRNA Control Vectors can also be used with any BLOCK-iT™ Pol II miR Validated miRNA Vector DuoPak (see page v) or your own miRNA vector constructed using the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit (see page v) as a positive control for easy analysis of knockdown in any mammalian cell line.

To use the Validated miRNA Control Vectors with the BLOCK-iT™ Pol II miR Validated miRNA Vector DuoPak, refer to the BLOCK-iT™ Pol II miR Validated miRNA Vector DuoPak manual for details. The manual is available from www.invitrogen.com or contact Technical Service (see page 32).

Continued on next page

Overview, Continued

Important

You will need appropriate reporter plasmids to successfully use the Validated miRNA Control Vectors. Reporter plasmids are not supplied with the Validated miRNA Control Vectors. You may purchase reporter plasmids or use existing reporter plasmids available in your laboratory. For details on the reporter plasmids, see page 7.

Types of Vectors

The following Validated miRNA Control Vectors are available from Invitrogen. The sense strand of the 21 nucleotide target sequence and gene specific information for each control vector is listed in the table below.

The 68 bp pre-miRNA insert sequence is listed on page 11 and the complete sequence for each vector is available for downloading from our web site at www.invitrogen.com.

Information	pcDNA™6.2-GW/EmGFP-miR-*lacZ*	pcDNA™6.2-GW/EmGFP-miR-*luc*	pcDNA™6.2-GW/EmGFP-miR-LMNA
Catalog no.	V49350-00	V49351-00	V49352-00
Gene Symbol	*lacZ*	*luc* (GL2)	LMNA
Species	*E. coli*	Firefly (*Photinus pyralis*)	Human
Primary Accession #	N/A	N/A	NM_170707 (variant 1 produces lamin A protein)
Coding Region	N/A	N/A	213-2207
21 nt Target Sequence (sense)	GACTACACAAATCAGCGATTT	TGAAACGATATGGGCTGAATA	GAAGGAGGAACTGGACTTCCA
Target Sequence Position	657-677 in coding region	200-220 in coding region	812-832

Advantages of the BLOCK-iT™ Pol II miR Validated miRNA Control Vectors

Use of the BLOCK-iT™ Pol II miR Validated miRNA Control Vectors for miRNA expression and RNAi analysis in mammalian cells provides the following advantages:

- Rapid and efficient way to perform knockdown experiments with guaranteed results
- Each pre-miRNA sequence is validated to knock down the target gene by at least 70%
- Permits visual or automated selection of cells expressing the pre-miRNA through co-cistronic expression of EmGFP (Emerald GFP)
- Allows **transient** or **stable** expression of miRNA in mammalian cells
- Gateway®-adapted vectors for easy recombination of the pre-miRNA sequence from the expression vector into the destination vector of choice for flexible miRNA expression options including viral expression and expression using tissue-specific or regulated promoters

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The BLOCK-iT™ Pol II miR RNAi Technology

The BLOCK-iT™ Pol II miR RNAi Technology is a next generation RNAi technology employing miRNA expression vectors that allow flexible expression of miRNA-based knockdown cassettes driven by RNA Polymerase II (Pol II) promoters in mammalian cells.

The BLOCK-iT™ Pol II miR Validated miRNA Control Vectors are specifically designed to allow expression of engineered pre-miRNA sequences and contain specific miR flanking sequences that allow proper processing of the miRNA. The expression vector design is based on the miRNA vector system developed in the laboratory of David Turner (U.S. Patent Publication No. 2004/0053876) and includes the use of endogenous murine miR-155 flanking sequences (see page 10 for details). The engineered miRNAs produced by the BLOCK-iT™ Pol II miR Validated miRNA Vectors fully complement their target site and cleave the target mRNA (see page 9 for details).

A variety of BLOCK-iT™ RNAi products are available from Invitrogen to facilitate RNAi analysis in mammalian and invertebrate systems. For more details on BLOCK-iT™ RNAi products, see the RNAi Central portal at www.invitrogen.com/rnai or contact Technical Service (see page 32).

Alternate Expression Systems

The pcDNA™6.2-GW/EmGFP-miR Validated miRNA Control Vectors express the pre-miRNA sequence in most mammalian cells at a high, constitutive level using the human cytomegalovirus (CMV) immediate early promoter.

If you wish to perform expression of the pre-miRNA in other systems such as tissue-specific, regulated, or lentiviral expression, the vectors allow easy recombination with other suitable destination vectors using the Gateway® Technology (see next page).

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Gateway® Technology

The Gateway® Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move your DNA sequence of interest into multiple vector systems.

The BLOCK-iT™ Pol II miR Validated miRNA Control Vectors are Gateway®-compatible, enabling the transfer of the pre-miRNA sequence into destination vectors for miRNA expression in viral systems or using regulated or tissue-specific promoter for expression.

To transfer the pre-miRNA sequence into a destination vector of choice using the BLOCK-iT™ Pol II miR Validated miRNA Control Vector and Gateway® Technology:

1. Generate an entry clone by performing a BP recombination reaction between the pcDNA™6.2-GW/EmGFP-miR Validated miRNA Control vector (expression clone) and a donor vector such as pDONR™221.
2. Then perform an LR recombination reaction between the resulting entry clone (pENTR™221/miR Validated entry clone) and a destination vector. See page 22 for more details.
3. Use the new expression clone to express the miRNA in mammalian cells.

For detailed information about the Gateway® Technology, refer to the Gateway® Technology with Clonase™ II manual which is available from our web site (www.invitrogen.com) or by contacting Technical Service (see page 32).

Purpose of this Manual

This manual provides an overview of the BLOCK-iT™ Pol II miR Validated miRNA Control Vectors and provides instructions and guidelines to:

- Transfect the pcDNA™6.2-GW/EmGFP-miR Validated miRNA Control Vector construct into mammalian cells for miRNA expression and RNAi analysis
- Generate stable cell lines, if desired
- Transfer the pre-miRNA sequence into a destination vector of choice using the Gateway® recombination reactions.
- Troubleshooting

Validated miRNA Control Vectors

Introduction

This section describes the preparation of Validated miRNA Control Vectors and features of the vectors. For details on the pre-miRNA sequence structure, see page 11.

Preparing Validated miRNA Control Vectors

The Validated miRNA Control Vectors are prepared as follows using the BLOCK-iT™ Pol II miR RNAi Technology (see page 3) and Gateway® Technology (see previous page):

- Invitrogen's RNAi Designer, an online tool, was used to design 21 nucleotide miRNA sequences for the target genes (*lacZ*, luciferase, and lamin A/C). Single-stranded oligonucleotides were ordered that contain the following design features and comprise the 68 bp pre-miRNA sequence (see page 11 for details on the structural features of the pre-miRNA):
  - Sequences encoding the miRNA of interest (stem and loop sequences)
  - Sequences required to facilitate directional cloning (overhangs) into pcDNA™6.2-GW/EmGFP-miR Expression Vector available from Invitrogen (see page v)
- The single-stranded oligos were annealed to generate the double-stranded oligo which was then ligated into the pcDNA™6.2-GW/EmGFP-miR Expression Vector using T4 DNA Ligase
- The ligation mixture was transformed into One Shot® TOP10 Chemically Competent *E coli* and the resulting transformants were analyzed by sequencing to confirm the insertion of the ds oligo insert.
- Clones were screened and validated as follows:
  - For *lacZ* and luciferase, the screening was performed using the *lacZ* or luciferase reporter genes
  - For lamin A/C, the screening was performed using BLOCK-iT™ RNAi Target Screening System (see page v) by performing an LR recombination reaction with the pSCREEN-iT™/*lacZ*-DEST Vector and an Ultimate™ ORF Entry Vector from Invitrogen corresponding to the lamin A/C gene.
- High-throughput transfection was performed with GripTite™ 293 MSR cell line, reporter gene (for *lacZ* and luciferase) or pSCREEN-iT™ expression clone (for lamin A/C), and the pcDNA™6.2-GW/EmGFP-miR Validated miRNA Control Vector using Lipofectamine™ 2000. After 24 hours, the expression of β-galactosidase and luciferase was analyzed using activity assays and quantitated. EmGFP fluorescence was also analyzed and quantitated using flow cytometry.
- A second screening was performed using similar conditions and the pre-miRNA sequences for each gene demonstrating at least 75% target gene knockdown in two separate experiments were selected for the final control vector.

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Validated miRNA Control Vectors, Continued

Features of pcDNA6.2-GW/EmGFP-miR-Validated miRNA Control Vector

The pcDNA™6.2-GW/EmGFP/miR Validated miRNA Control Vector contains the following features:

- Human CMV promoter for high-level, constitutive expression of the miRNA from an RNA Polymerase II-dependent promoter
- 5′ and 3′ miR flanking regions for formation of an engineered pre-miRNA
- Contains a pre-miRNA sequence encoded within the 68 bp insert sequence (see page 11 for details) targeting the gene of interest
- Emerald Green Fluorescent Protein (EmGFP) coding sequence for co-cistronic expression with the pre-miRNA (see page 12 for EmGFP)
- Two recombination sites, *att*B1 and *att*B2, flanking the pre-miRNA expression cassette for recombinational cloning of the pre-miRNA expression cassette into a Gateway® destination vector
- Herpes Simplex virus (HSV) thymidine kinase (TK) polyadenylation signal for termination and polyadenylation of the transcript
- Spectinomycin resistance gene for selection in *E. coli*
- pUC origin for high-copy maintenance of the plasmid in *E. coli*
- Blasticidin resistance gene for selection in *E. coli* and mammalian cells to generate cell lines stably expressing the miRNA

For a map of the pcDNA™6.2-GW/EmGFP/miR Validated miRNA Control Vector, see page 29.

Negative Control

The Validated miRNA Control Vectors also include a negative control plasmid.

The pcDNA™6.2-GW/EmGFP-miR-neg control plasmid contains an insert that can form a hairpin structure that is processed into mature miRNA, but is not predicted to target any known vertebrate gene. Thus, this plasmid serves as a suitable negative control for pre-miRNA experiments with Validated miRNA Control Vectors.

The negative control sequence without overhangs is shown below, the target sequence is underlined (for a map of the vector, see page 31):

5′-GAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT-3′

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Reporter Plasmids

You will need appropriate reporter plasmids to successfully use the Validated miRNA Control Vectors. The reporter plasmid is co-transfected with the Validated miRNA Control Vector into mammalian cells and provides a means to assess the RNAi response in your cell line by assaying for knockdown of the target gene using suitable assay methods.

Reporter plasmids are not supplied with the Validated miRNA Control Vectors. You may purchase reporter plasmids or use existing reporter plasmids available in your laboratory. See below for details on the reporter plasmids.

Control Vector	Reporter Plasmid
pcDNA™6.2-GW/EmGFP-miR-*lacZ*	Expressing β-galactosidase (*lacZ*)
pcDNA™6.2-GW/EmGFP-miR-*luc*	Expressing luciferase (GL2 type)
pcDNA™6.2-GW/EmGFP-miR-LMNA	Not required if you are using endogenous lamin (see below)

For details on using the reporter plasmids, see page 18.

Endogenous Lamin A/C Expression

If you are performing knockdown of the endogenous lamin, you will need a cell line that expresses the human lamin A/C gene (*e.g.* A549, HeLa, HEK 293, HT1080, COS-7).

Note: The pcDNA™6.2-GW/EmGFP-miR-LMNA Validated miRNA construct expresses an miRNA targeted to the human lamin A/C gene. If you are using a non-human cell line, the lamin A/C gene may contain mismatches in the target region that can render the miRNA inactive.

If you are using a cell line that does not express lamin A/C, be sure to use a reporter plasmid expressing lamin A/C.

Important

Confirm that the pre-miRNA target site is present in your reporter plasmid prior to performing the knockdown experiments. See page 2 for the 21 nucleotide sense target sequence.

Using miRNA for RNAi Analysis

Introduction

RNA interference (RNAi) describes the phenomenon by which short, homologous RNA duplexes induce potent and specific inhibition of eukaryotic gene expression via the degradation of complementary messenger RNA (mRNA), and is functionally similar to the processes of post-transcriptional gene silencing (PTGS) or cosuppression in plants (Cogoni *et al.*, 1994; Napoli *et al.*, 1990; Smith *et al.*, 1990; van der Krol *et al.*, 1990) and quelling in fungi (Cogoni & Macino, 1997; Cogoni & Macino, 1999; Romano & Macino, 1992).

In plants, the PTGS response is thought to occur as a natural defense against viral infection or transposon insertion (Anandalakshmi *et al.*, 1998; Jones *et al.*, 1998; Li & Ding, 2001; Voinnet *et al.*, 1999). In experimental settings, RNAi is widely used to silence genes through transfection of RNA duplexes or introduction of vector-expressed short hairpin RNA (shRNA).

The RNAi Pathway

In eukaryotic organisms, dsRNA produced *in vivo*, introduced by pathogens, or through research, is processed into 21-23 nucleotide double-stranded short interfering RNA duplexes (siRNA) by an enzyme called Dicer, a member of the RNase III family of double-stranded RNA-specific endonucleases (Bernstein *et al.*, 2001; Ketting *et al.*, 2001).

Each siRNA then incorporates into an RNA-induced silencing complex (RISC), an enzyme complex that serves to target cellular transcripts complementary to the siRNA for specific cleavage and degradation, or translational repression (Hammond *et al.*, 2000; Nykanen *et al.*, 2001). MicroRNAs (miRNAs) are endogenous RNAs that trigger gene silencing (Ambros, 2001; Carrington & Ambros, 2003).

miRNA Pathway

MicroRNAs (miRNAs) are endogenously expressed small ssRNA sequences of ~22 nucleotides in length which naturally direct gene silencing through components shared with the RNAi pathway (Bartel, 2004). Unlike shRNAs, however, the miRNAs are found embedded, sometimes in clusters, in long primary transcripts (pri-miRNAs) of several kilobases in length containing a hairpin structure and driven by RNA Polymerase II (Pol II) (Lee *et al.*, 2004), the polymerase also responsible for mRNA expression.

Drosha, a nuclear RNase III, cleaves the stem-loop structure of the pri-miRNA to generate small hairpin precursor miRNAs (pre-miRNAs) which are ~70 nucleotides in length (Zeng *et al.*, 2005). The pre-miRNAs are exported from the nucleus to the cytoplasm by exportin-5, a nuclear transport receptor(Bohnsack *et al.*, 2004; Yi *et al.*, 2003). Following the nuclear export, the pre-miRNAs are processed by Dicer into a ~22 nucleotides miRNA (mature miRNA) molecule, and incorporated into an miRNA-containing RNA-induced silencing complex (miRISC) (Cullen, 2004).

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Translational Repression versus Target Cleavage

The mature miRNAs regulate gene expression by mRNA cleavage (mRNA is nearly complementary to the miRNA) or translational repression (mRNA is not sufficiently complementary to the miRNA). Target cleavage can be induced artificially by altering the target or the miRNA sequence to obtain complete hybridization (Zeng *et al.*, 2002).

In animals, most miRNAs imperfectly complement their targets and interfere with protein production without directly inducing mRNA degradation (Ambros, 2004). Nonetheless, these miRNAs are found associated with the RNAi nuclease AGO2 (Liu *et al.*, 2004; Meister *et al.*, 2004), and at least two miRNAs with close matches to their target sequences, particularly in their 5′ regions, have been shown to cleave cognate mRNAs (Yekta *et al.*, 2004; Yu *et al.*, 2005).

The engineered miRNAs produced by the BLOCK-iT™ Pol II miR Validated miRNA Control Vectors (see below) fully complement their target site and cleave the target mRNA. Sequence analysis showed that the primary cleavage site at the phosphodiester bond in the mRNA found opposite the tenth and eleventh bases of the engineered miRNA as predicted for RNAi-mediated cleavage (Elbashir *et al.*, 2001) similar to siRNA mediated cleavage.

Using a Vector-Based System to Express Engineered miRNA

Use of siRNA (diced siRNA or synthetic siRNA) for RNAi analysis in mammalian cells is limited by their transient nature. To address these limitations, a number of groups have developed vector-based systems to facilitate expression of engineered short hairpin RNA (shRNA) sequences in mammalian cells using Pol III promoters (Brummelkamp *et al.*, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; Sui *et al.*, 2002; Yu *et al.*, 2002). However, the use of shRNA vectors for RNAi analysis requires the screening of large number of sequences to identify active sequences and the use of Pol III promoters limits applications such as tissue-specific expression.

To overcome limitations with siRNA and shRNA, we have developed Gateway®-adapted expression vectors that enable the expression of engineered miRNA sequences from Pol II promoters. The pcDNA™6.2-GW/EmGFP-miR-Validated miRNA Control Vectors contain a ds oligo encoding a pre-miRNA sequence (see page 11). The resulting expression construct may be introduced into mammalian cells for transient expression of the miRNA sequence, or stable transfectants can be generated. If desired, the pre-miRNA sequence may be easily and efficiently transferred into other suitable destination vector by Gateway® recombination reactions (see page 4).

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Using miRNA for RNAi Analysis, Continued

Human CMV Promoter

The Validated miRNA Control Vectors contain the human cytomegalovirus (CMV) immediate early promoter to allow high-level, constitutive miRNA expression in mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).

We have chosen the human CMV promoter to control vector-based expression of miRNA molecules in mammalian cells for the following reasons:

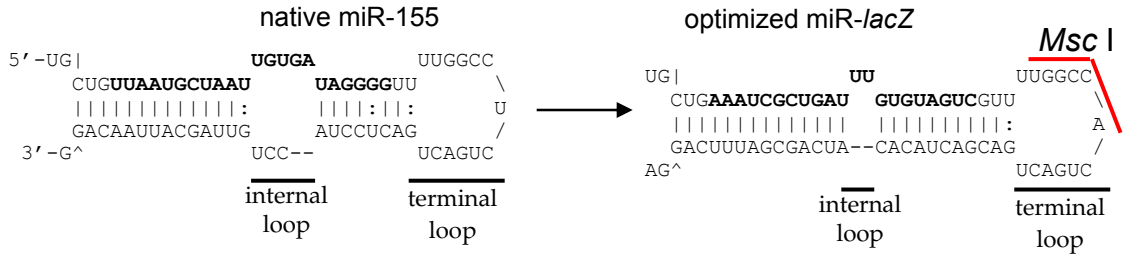
- The promoter is recognized by RNA Polymerase II and controls high-level, constitutive expression of miRNA and co-cistronic reporter genes
- The promoter is active in most mammalian cell types

Note: Although highly active in most mammalian cell lines, activity of the viral CMV promoter can be down-regulated in some cell lines due to methylation (Curradi *et al.*, 2002), histone deacetylation (Rietveld *et al.*, 2002), or both.

Design of the Engineered Pre-miRNA

The engineered pre-miRNA sequence structure is based on the murine miR-155 sequence (Lagos-Quintana *et al.*, 2002). The 5′ and 3′ flanking regions derived from the miR-155 transcript were inserted in the vector to aid in the processing of the engineered pre-miRNA sequence. We optimized the stem-loop structure and a 2 nucleotide internal loop results in higher knockdown rate than the 5 nucleotide/3 nucleotide internal loop found in native miR-155 molecule. An *Msc* I site was incorporated in the terminal loop to aid in sequence analysis.

The changes made to the native miR-155 to form an engineered pre-miRNA directed against *lacZ* (targeting sequence in bold) are shown below.



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Structure of the Engineered Pre-miRNA

The pcDNA™6.2-GW/EmGFP-miR-Validated miRNA Control Vectors contain a 68 bp engineered pre-miRNA sequence targeting *lacZ*, luciferase, or lamin A/C.

For optimized knockdown results, the following structural features were incorporated in the ds oligo encoding the engineered pre-miRNA:

- A 4 nucleotide, 5′ overhang (TGCT) complementary to the vector (required for directional cloning)
- A 5′G + short 21 nucleotide antisense sequence (mature miRNA) derived from the target gene, followed by
- A short spacer of 19 nucleotides to form the terminal loop and
- A short sense target sequence with 2 nucleotides removed (Δ2) to create an internal loop
- A 4 nucleotide, 5′ overhang (CAGG) complementary to the vector (required for directional cloning)

The structural features are depicted in the figure below. The 68 bp sequence for each control vector is listed below.

Engineered Pre-miRNA Sequences

The 68 bp engineered pre-miRNA sequence targeting *lacZ*, luciferase, or lamin A/C are listed below. The target sequence is underlined. The complete sequence of the control vector is available for downloading from our web site at www.invitrogen.com.

LacZ pre-miRNA

TGCTGAAATCGCTGATTTGTGTAGTCGTTTTGGCCACTGACTGACGACTACACATCAGCGATTTCAGG

Luciferase pre-miRNA

TGCTGTATTCAGCCCATATCGTTTCAGTTTTGGCCACTGACTGACTGAAACGATGGGCTGAATACAGG

Lamin A/C pre-miRNA

TGCTGTGGAAGTCCAGTTCCTCCTTCGTTTTGGCCACTGACTGACGAAGGAGGCTGGACTTCCACAGG

Pre-miRNA Expression Cassette

The engineered pre-miRNA sequence in pcDNA™6.2-GW/EmGFP-miR Validated miRNA Control Vector is flanked on either side with sequences from murine miR-155 to allow proper processing of the engineered pre-miRNA sequence (see page 15 for flanking region sequences).

The pre-miRNA sequence, adjacent miR-155 flanking regions, and EmGFP coding sequence are denoted as the pre-miRNA expression cassette and is shown below. This expression cassette is transferred between vectors during Gateway® recombination reactions.

Once the engineered pre-miRNA expression cassette is introduced into the mammalian cells for expression, the pre-miRNA forms an intramolecular stem-loop structure similar to the structure of endogenous pre-miRNA that is then processed by the endogenous Dicer enzyme into a 22 nucleotide mature miRNA.

Note: The 21 nucleotides are derived from the target sequence while the 3′ most nucleotide is derived from the native miR-155 sequence (see figure on page 15).

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Green Fluorescent Protein

Description

The BLOCK-iT™ Pol II miR Validated miRNA Control Vectors combine the ease and flexibility of Gateway® expression vectors with the brightness of Emerald Green Fluorescent Protein (EmGFP) derived from *Aequorea victoria* GFP.

After transfection of the expression clone into mammalian cells, the fluorescent EmGFP can be identified by fluorescence detection methods. Since the EmGFP is expressed co-cistronically with the miRNA, there is a strong correlation of EmGFP expression with miRNA knockdown activity allowing you to visually track the cells in which knockdown is occurring or sort the cells using a flow cytometer.

Green Fluorescent Protein (GFP)

Green Fluorescent Protein (GFP) is a naturally occurring bioluminescent protein derived from the jellyfish *Aequorea victoria* (Shimomura *et al.*, 1962). GFP emits fluorescence upon excitation, and the gene encoding GFP contains all of the necessary information for posttranslational synthesis of the luminescent protein. GFP is often used as a molecular beacon because it requires no species-specific cofactors for function, and the fluorescence is easily detected using fluorescence microscopy and standard filter sets. GFP can function as a reporter gene downstream of a promoter of interest and upstream of one or more pre-miRNAs.

GFP and Spectral Variants

Modifications have been made to the wild-type GFP to enhance its expression in mammalian systems. These modifications include amino acid substitutions that correspond to the codon preference for mammalian use, and mutations that increase the brightness of the fluorescence signal, resulting in "enhanced" GFP (Zhang *et al.*, 1996). Mutations have also arisen or have been introduced into GFP that further enhance and shift the spectral properties of GFP such that these proteins will emit fluorescent color variations (reviewed in Tsien, 1998). The Emerald GFP (EmGFP) is a such variant of enhanced GFP.

Note

We have observed reduced EmGFP expression from miRNA-containing vectors due to processing of the transcripts. In most cases, EmGFP expression should remain detectable.

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Green Fluorescent Protein, Continued

EmGFP

The EmGFP variant has been described in a published review (Tsien, 1998) and is summarized below. The amino acid mutations are represented by the single letter abbreviation for the amino acid in the consensus GFP sequence, followed by the codon number and the single letter amino acid abbreviation for the substituted amino acid.

Fluorescent Protein	GFP Mutations*
EmGFP	S65T, S72A, N149K, M153T, I167T

*Mutations listed are as described in the literature. When examining the actual sequence, the vector codon numbering starts at the first amino acid **after** the initiation methionine of the fluorescent protein, so that mutations appear to be increased by one position. For example, the S65T mutation actually occurs in codon 66 of EmGFP.

EmGFP Fluorescence

The fluorescent protein from the Validated miRNA vectors have the following excitation and emission wavelengths, as published in the literature (Tsien, 1998):

Excitation (nm)	Emission (nm)
487	509

Filter Sets for Detecting EmGFP Fluorescence

The EmGFP can be detected with standard FITC filter sets. However, for optimal detection of the fluorescence signal, you may use a filter set which is optimized for detection within the excitation and emission ranges for the fluorescent protein. The filter set for fluorescence microscopy and the manufacturer are listed below:

Filter Set	Manufacturer
Omega XF100	Omega (www.omegafilters.com)

Methods

Using the Validated miRNA Control Vectors

Introduction

General guidelines for using the BLOCK-iT™ Pol II miR Validated miRNA Control vectors (Validated miRNA Control Vectors) are described in this section.

For transfecting the Validated miRNA Control plasmids in a mammalian cell line of choice for miRNA expression, see page 16.

Note

A sufficient amount of highly pure plasmid DNA is included with each Validated miRNA Control Vector to allow you to perform transfection experiments directly without propagating the plasmid. If you wish to propagate the plasmid, see below.

Propagating Validated miRNA Control Vectors

You may use any suitable *rec*A, *end*A *E. coli* strain for propagating and maintaining the Validated miRNA Control Vectors. We recommend using One Shot® TOP10 Chemically Competent *E. coli* (see page v) available from Invitrogen for transformation.

To maintain the integrity of the vector, select for transformants in LB media containing 50 µg/ml spectinomycin. Spectinomycin is available from Sigma (see page v). See page 27 for preparing spectinomycin.

If you wish to use Blasticidin for selection, use Low Salt LB containing 100 µg/ml Blasticidin to grow transformants. See page 27 for a recipe of Low Salt LB containing Blasticidin.

Plasmid Preparation

After transformation, you may isolate plasmid DNA using any plasmid DNA isolation method. To obtain highly pure plasmid DNA suitable for mammalian cell transfections, we recommend isolating plasmid DNA using the PureLink™ Plasmid Purification Kits (see page v).

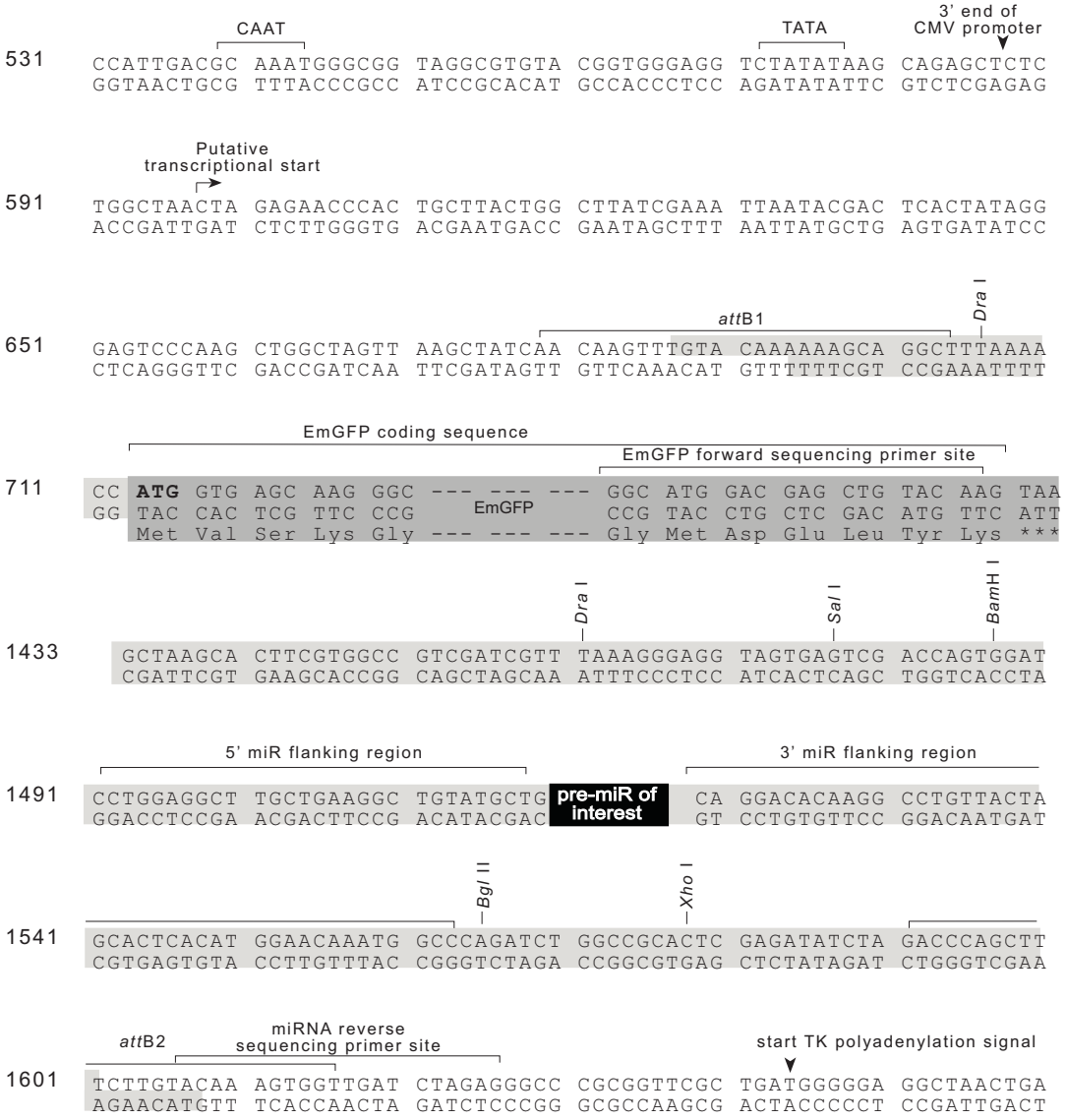
You will need ~2-5 µg plasmid DNA for each experiment.

Continued on next page

Pre-miRNA Insert Region of pcDNA™6.2-GW/EmGFP-miR Validated miRNA Control Vector

Below is the region of pcDNA6.2™-GW/EmGFP-miR Validated miRNA Control Vector showing the pre-miRNA expression cassette along with the *att*B sites. The dark shaded region represents the EmGFP coding sequence.

Note: If you are performing Gateway® recombination reactions, the light shaded regions correspond to those DNA sequences transferred from pcDNA6.2™-GW/EmGFP-miR Validated miRNA Control Vector into the Gateway® destination vector following recombination. The resulting expression clone after Gateway® recombination contains a pre-miRNA expression cassette consisting of the EmGFP coding sequence, 5′ miR flanking region, pre-miRNA sequence, and 3′ miR flanking region as shown below.


CAAT
TATA
3′ end of CMV promoter
531
CCATTGACGC AAATGGGCGG TAGGCGTGTA CGGTGGGAGG TCTATATAAG CAGAGCTCTC
GGTAACTGCG TTTACCCGCC ATCCGCACAT GCCACCCTCC AGATATATTC GTCTCGAGAG
Putative transcriptional start
591
TGGCTAACTA GAGAACCCAC TGCTTACTGG CTTATCGAAA TTAATACGAC TCACTATAGG
ACCGATTGAT CTCTTGGGTG ACGAATGACC GAATAGCTTT AATTATGCTG AGTGATATCC
attB1
Dra I
651
GAGTCCCAAG CTGGCTAGTT AAGCTATCAA CAAGTTTGTA CAAAAAAGCA GGCTTTAAAA
CTCAGGGTTC GACCGATCAA TTCGATAGTT GTTCAAACAT GTTTTTTCGT CCGAAATTTT
EmGFP coding sequence
EmGFP forward sequencing primer site
711
CC ATG GTG AGC AAG GGC --- --- --- GGC ATG GAC GAG CTG TAC AAG TAA
GG TAC CAC TCG TTC CCG EmGFP CCG TAC CTG CTC GAC ATG TTC ATT
Met Val Ser Lys Gly --- --- --- Gly Met Asp Glu Leu Tyr Lys ***
Dra I
Sal I
BamH I
1433
GCTAAGCA CTTCGTGGCC GTCGATCGTT TAAAGGGAGG TAGTGAGTCG ACCAGTGGAT
CGATTCGT GAAGCACCGG CAGCTAGCAA ATTTCCCTCC ATCACTCAGC TGGTCACCTA
5′ miR flanking region
3′ miR flanking region
1491
CCTGGAGGCT TGCTGAAGGC TGTATGCTG pre-miR of interest CA GGACACAAGG CCTGTTACTA
GGACCTCCGA ACGACTTCCG ACATACGAC GT CCTGTGTTCC GGACAATGAT
Bgl II
Xho I
1541
GCACTCACAT GGAACAAATG GCCCAGATCT GGCCGCACTC GAGATATCTA GACCCAGCTT
CGTGAGTGTA CCTTGTTTAC CGGGTCTAGA CCGGCGTGAG CTCTATAGAT CTGGGTCGAA
attB2
miRNA reverse sequencing primer site
start TK polyadenylation signal
1601
TCTTGTACAA AGTGGTTGAT CTAGAGGGCC CGCGGTTCGC TGATGGGGGA GGCTAACTGA
AGAACATGTT TCACCAACTA GATCTCCCGG GCGCCAAGCG ACTACCCCCT CCGATTGACT

Continued on next page

Transfecting Cells

Introduction

This section provides general guidelines to transfect the Validated miRNA Control Vector into the mammalian cell line of interest to perform transient RNAi analysis. Performing transient RNAi analysis is useful to quickly screen for an RNAi response in your mammalian cell line

If you want to generate a stable cell line expressing the miRNA, see page 20.

Factors Affecting Gene Knockdown Levels

A number of factors can influence the degree to which expression of your gene of interest is reduced (*i.e.* gene knockdown) in an RNAi experiment including:

- Transfection efficiency
- Transcription rate of the target gene of interest
- Stability of the target protein
- Growth characteristics of your mammalian cell line

Take these factors into account when designing your RNAi experiments.

Plasmid Preparation

You may use the supplied highly pure plasmid DNA supplied with the vectors for transfection experiments.

If you need more plasmid DNA, you need to propagate the vectors (see page 14). Plasmid DNA for transfection into eukaryotic cells must be very clean and free from contamination with phenol or sodium chloride. Contaminants kills the cells, and salt interferes with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink™ Plasmid Purification Kits (see page v) or CsCl gradient centrifugation.

Reporter Plasmids

You will need appropriate reporter plasmids to successfully use the Validated miRNA Control Vectors. Reporter plasmids are not supplied with the Validated miRNA Control Vectors. You may purchase reporter plasmids or use existing reporter plasmids available in your laboratory. For details on the reporter plasmids, see page 7.

Note

For knockdown experiments using endogenous lamin, use a cell line that expresses the human lamin A/C gene (*e.g.* A549, HeLa, HEK 293, HT1080, COS-7).

If you are using a cell line that does not express lamin A/C, be sure to use a reporter plasmid expressing lamin A/C.

Continued on next page

Transfecting Cells, Continued

Methods of Transfection

For established cell lines (*e.g.* COS, HEK-293), consult original references or the supplier of your cell line for the optimal method of transfection. Pay particular attention to media requirements, when to pass the cells, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Methods for transfection include calcium phosphate (Chen & Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner & Ringold, 1989), and electroporation (Chu *et al.*, 1987; Shigekawa & Dower, 1988). Choose the method and reagent that provides the highest efficiency transfection in your mammalian cell line. For a recommendation, see below.

For high-efficiency transfection in a broad range of mammalian cell lines, we recommend using the cationic lipid-based Lipofectamine™ 2000 Reagent (see page v) available from Invitrogen (Ciccarone *et al.*, 1999). Using Lipofectamine™ 2000 to transfect plasmid DNA into eukaryotic cells offers the following advantages:

- Provides the highest transfection efficiency in many mammalian cell types.
- DNA-Lipofectamine™ 2000 complexes can be added directly to cells in culture medium in the presence of serum.
- Removal of complexes, medium change, or medium addition following transfection are not required, although complexes can be removed after 4-6 hours without loss of activity.

For more information on Lipofectamine™ 2000 Reagent, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 32). Specific transfection protocols for various cell lines using Lipofectamine™ 2000 are available on our web site at www.invitrogen.com.

Negative Control

As **negative control**, perform parallel transfections with the pcDNA™6.2-GW/EmGFP-miR-neg control plasmid supplied with the Validated miRNA Control Vectors.

Continued on next page

Transfecting the Control Vectors and Reporter Plasmids

For *lacZ* or luciferase knockdown experiments, perform co-transfection experiments as follows:

- Co-transfect pcDNA™6.2-GW/EmGFP-miR-*lacZ* plasmid and reporter plasmid expressing β-galactosidase into your mammalian cell line. For optimal results, we recommend using 3-fold more Validated miRNA Control plasmid DNA than reporter plasmid DNA in the co-transfection (see example below).
- Co-transfect the pcDNA™6.2-GW/EmGFP-miR-*luc* plasmid and reporter plasmid expressing full length firefly (*Photinus pyralis*) luciferase into your mammalian cell line. For optimal results, we recommend using 3-fold more Validated miRNA Control plasmid DNA than reporter plasmid DNA in the co-transfection (see example below)

For example, use 300 ng of pcDNA™6.2-GW/miR-*lacZ* DNA and 100 ng of reporter plasmid DNA when transfecting cells plated in a 24-well format.

For endogenous lamin knockdown experiments, transfect **only** pcDNA™6.2-GW/EmGFP-miR-LMNA plasmid into your mammalian cells expressing the human lamin A/C gene. There is no need to use a reporter plasmid.

If you are using a cell line that does not express human lamin A/C, then you will need to co-transfect the cells with a reporter plasmid expressing lamin A/C.

Assaying for Expression

To perform RNAi analysis using the control vectors and reporter plasmids, you need to assay for expression as described below:

- If you are using pcDNA™6.2-GW/EmGFP-miR-*lacZ* with a reporter plasmid expressing full-length β-galactosidase, assay for β-galactosidase expression by western blot analysis using β-gal Antiserum (Catalog no. R901-25), by activity assay using FluoReporter® *lacZ*/Galactosidase Quantitation Kit (Catalog no. F-2905), or by staining the cells for activity using the β-Gal Staining Kit (Catalog no. K1465-01).
- If you are using pcDNA™6.2-GW/EmGFP-miR-*luc* with a reporter plasmid expressing full-length firefly luciferase (GL2 type), assay for luciferase expression by activity assay using a luminescence assay.
- If you are using pcDNA™6.2-GW/EmGFP-miR-LMNA, assay for the endogenous lamin expression by western blot analysis or immunofluorescence.
- For visualization of EmGFP, see the next page.

Generating Stable Cell Lines

Once you have performed transient RNAi experiments with the Validated miRNA Control Vectors, you may wish to establish a stable cell line that constitutively expresses the miRNA for stable RNAi experiments. See page 20 for details on generating stable cell lines using Blasticidin selection.

Detecting Fluorescence

Introduction

You can perform analysis of the EmGFP fluorescent protein from the expression clone in either transiently transfected cells or stable cell lines. Once you have transfected your expression clone into mammalian cells, you may detect EmGFP protein expression directly in cells by fluorescence microscopy or other methods that use light excitation and detection of emission. The EmGFP expression is strongly correlated with the expression of your miRNA.

See below for recommended fluorescence microscopy filter sets.

Filters for Use with EmGFP

The EmGFP can be detected with standard FITC filter sets. However, for optimal detection of the fluorescence signal, you may use a filter set which is optimized for detection within the excitation and emission ranges for the fluorescent protein such as the Omega XF100 filter set for fluorescence microscopy. The spectral characteristics of EmGFP are listed in the table below:

Excitation (nm)	Emission (nm)
487	509

For information on obtaining these filter sets, contact Omega Optical, Inc. (www.omegafilters.com) or Chroma Technology Corporation (www.chroma.com).

Fluorescence Microscope

You may view the fluorescence signal of EmGFP in cells using an inverted fluorescence microscope with FITC filter or Omega XF100 filter (available from www.omegafilters.com) for viewing cells in culture or a flow cytometry system.

Color Camera

If desired, you may use a color camera that is compatible with the microscope to photograph the cells. We recommend using a digital camera or high sensitivity film, such as 400 ASA or greater.

Detecting Transfected Cells

After transfection, allow the cells to recover for 24 to 48 hours before assaying for fluorescence. Medium can be removed and replaced with PBS during viewing to avoid any fluorescence due to the medium. Be sure to replace PBS with fresh medium if you wish to continue growing the cells.

Note: Cells can be incubated further to optimize expression of EmGFP.

What You Should See

Cells expressing EmGFP will appear brightly labeled and will emit a green fluorescence signal that should be easy to detect above the background fluorescence. **Note:** The fluorescence signal of EmGFP from miRNA-containing vectors is reduced when compared to non-miRNA containing vectors.

Cells with bright fluorescence will demonstrate highest knockdown. However, cells with reduced fluorescence may still express the miRNA and demonstrate knockdown since the expression levels required to observe gene knockdown are generally lower than that required to detect EmGFP expression.

Generating a Stable Cell Line

Introduction

Guidelines for generating cell lines stably expressing the miRNA are included in this section.

Blasticidin

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965). Resistance is conferred by expression of either one of two blasticidin *S deaminase* genes: *bsd* from *Aspergillus terreus* (Kimura *et al.*, 1994) or *bsr* from *Bacillus cereus* (Izumi *et al.*, 1991). These deaminases convert blasticidin S to a nontoxic deaminohydroxy derivative (Izumi *et al.*, 1991). Blasticidin is available separately from Invitrogen (see page v for ordering information). For information on preparing and handling Blasticidin see page 27.

You will select for stable cells using blasticidin. This requires a minimum of 10-12 days after transfection, but allows generation of clonal cell lines that stably express the miRNA sequence.

Materials Needed

You will need the following materials:

- Mammalian cell line of interest (make sure that cells are healthy and > 90% viable before beginning)
- pcDNA™6.2-GW/EmGFP-miR Validated Control expression constructs
- pcDNA™6.2-GW/EmGFP-miR-neg Control Plasmid
- Transfection reagent of choice (*e.g.* Lipofectamine™ 2000)
- Blasticidin (see page v for ordering information and page 27 to prepare Blasticidin)
- Appropriate tissue culture dishes and supplies

Determining Blasticidin Sensitivity

To successfully generate a stable cell line expressing the miRNA, you first need to determine the minimum concentration of Blasticidin required to kill your untransfected host cell line. Most mammalian cells are killed by 2-10 µg/ml Blasticidin. Test a range of concentrations to ensure that you determine the minimum concentration necessary for your cell line (see protocol below). Refer to page 28 for instructions on how to prepare and store Blasticidin.

1. Prepare 6 plates of cells so that each plate will be approximately 25% confluent.
2. Replace the growth medium with fresh growth medium containing a range of Blasticidin concentrations: 0, 1, 3, 5, 7.5, and 10 µg/ml.
3. Replenish the selective media every 3-4 days, and observe the percentage of surviving cells.
4. Count the number of viable cells at regular intervals to determine the appropriate concentration of antibiotic that kills your cells within 1-3 weeks after addition of Blasticidin.

Continued on next page

Generating a Stable Cell Line, Continued

Guidelines for Transfection and Selection

Once you have determined the appropriate Blasticidin concentration to use for selection, you can generate a stable cell line expressing your Validated miRNA Control expression construct.

1. Transfect the mammalian cell line of interest with the pcDNA™6.2-GW/EmGFP-miR control expression construct using your transfection method of choice.
2. 24 hours after transfection, wash the cells and add fresh growth medium without Blasticidin.
3. 48 hours after transfection, split the cells into fresh growth medium without Blasticidin such that they are no more than 25% confluent. If the cells are too dense, the antibiotic will not kill the cells. Antibiotics work best on actively dividing cells.
4. Incubate the cells at 37°C for 2-3 hours until they have attached to the culture dish.
5. Remove the growth medium and replace with fresh growth medium containing Blasticidin at the predetermined concentration required for your cell line (see previous page).
6. Feed the cells with selective media every 3-4 days until Blasticidin-resistant colonies can be identified.
7. Pick at least 5 Blasticidin-resistant colonies and expand each clone to assay for knockdown of the target gene.
8. Assay for target gene knockdown, compare to uninduced cells and cells stably transfected with pcDNA™6.2-GW/EmGFP-miR-neg Control Plasmid.

Transferring the Pre-miRNA Expression Cassette to Destination Vectors

Introduction

The Validated miRNA Control Vectors contain *att* sites to facilitate the transfer of the pre-miRNA expression cassette into appropriate Gateway® destination vectors to allow for expression of the miRNA in viral systems or using tissue-specific promoters.

The pre-miRNA is transcribed by RNA Polymerase II (Pol II); the pre-miRNA expression cassette can be transferred to other Gateway® adapted destination vectors utilizing Pol II promoters, which allows expression of the pre-miRNA.

Important

Since the pcDNA™6.2-GW/EmGFP-miR Validated miRNA Control Vectors contain *att*B sites, the expression vectors containing the pre-miRNA expression cassette **cannot** be used directly with a destination vector to perform the LR recombination reaction.

Transferring the Cassette

To transfer the pre-miRNA expression cassette into other destination vectors, you need to first generate an entry clone containing *att*L sites by performing a BP recombination reaction, then use the resulting entry clone in an LR recombination reaction with a destination vector containing *att*R sites to generate a new miRNA expression clone.

The transfer of the miRNA sequence into the destination vector can be performed using the standard BP and LR recombination reactions or Rapid BP/LR recombination reactions as described on the next page.

See below for an overview of the Gateway® recombination reactions and page 15 for the recombination region.

Gateway® Recombination Reactions

Two recombination reactions constitute the basis of the Gateway® Technology. You will perform both Gateway® recombination reactions to transfer the pre-miRNA expression cassette from pcDNA™6.2-GW/EmGFP-miR Control Vector to a new destination vector as outlined below.

BP Reaction

Facilitates recombination of an *att*B substrate (like a linearized *att*B expression clone) with an *att*P substrate (donor vector) to create an *att*L-containing entry clone. This reaction is catalyzed by BP Clonase™ II enzyme mix.

You will recombine pcDNA™6.2-GW/EmGFP-miR Validated miRNA Control Vector (expression clone, *att*B substrate) with a *att*P substrate (donor vector such as pDONR™221) first to form an entry clone.

LR Reaction

Facilitates recombination of an *att*L substrate (entry clone) with an *att*R substrate (destination vector) to create an *att*B-containing expression clone. This reaction is catalyzed by LR Clonase™ enzyme mix.

The resulting entry clone (*att*L substrate) from the BP reaction is then recombined with the destination vector (*att*R substrate) to form a new miRNA expression clone.

Continued on next page

Transferring the Pre-miRNA Expression Cassette to Destination Vectors, Continued

Choosing a Suitable Protocol

Based on your experimental needs, you may choose between the standard or Rapid BP/LR recombination reactions as described in the table below:

If You Wish to….	Then Choose…..	Described
Generate the expression clones using a fast protocol but obtain fewer (~10% of the total number of clones) expression clones than the standard protocol	Rapid BP/LR Recombination Protocol	In the BLOCK-iT™ Pol II miR Validated miRNA Vector DuoPak manual (download from www.invitrogen.com or contact Technical Service, page 32).
Maximize the number of expression clones generated and isolate entry clones for future use	Standard BP and LR Protocols	In the Gateway® Technology with Clonase™ II manual (download from www.invitrogen.com or contact Technical Service, page 32).

Donor Vector

A large variety of donor vectors are available from Invitrogen (see page v). We recommend using pDONR™221 vector.

Appropriate Destination Vectors

A large selection of Gateway® destination vectors are available from Invitrogen. The various Gateway® vectors have widely different transcriptional and technical properties, which can be used to express the pre-miRNA. They offer custom promoter cloning, tissue-specific expression, regulated expression, and lentiviral transduction of the pre-miRNA. In addition, destination vectors providing N-terminal reporter genes can be used after removal of EmGFP.

A list of Gateway® destination vectors that are compatible with the Validated miRNA Control Vectors is shown below. For more details, visit www.invitrogen.com or contact Technical Service (see page 32).

Destination Vector	Catalog No.
Standard Destination Vectors	
pLenti6/V5-DEST	V496-10
pLenti6/UbC/V5-DEST	V499-10
pEF-DEST51	12285-011
pT-REx™-DEST30	12301-016
pEF5/FRT/V5-DEST™ (Flp-In™)	V6020-20
N-terminal reporter tag vectors, *e.g.*:	
pcDNA™6.2/nGeneBLAzer™-DEST	12578-068, 12578-050
pcDNA™6.2/N-YFP-DEST	V358-20
Multisite Gateway® Destination Vectors	
pDEST™/R4-R3	12567-023
pLenti6/R4R2/V5-DEST	K591-10

Continued on next page

Transferring the Pre-miRNA Expression Cassette to Destination Vectors, Continued

Important

Transferring the pre-miRNA expression cassette from pcDNA™6.2-GW/EmGFP-miR Validated miRNA Control Vector to the pLenti6/BLOCK-iT™-DEST destination vector will not yield a functional miRNA expression vector because this vector does not carry a Pol II promoter upstream of the *att*R1 site. **Expression of the pre-miRNA requires the destination vector to supply a Pol II promoter.**

For Lentiviral expression, transfer to pLenti6/V5-DEST as described in the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System manual, available for downloading from our web site (www.invitrogen.com) or by contacting Technical Service (see page 32).

Performing the BP and LR Recombination Reactions

To perform the Rapid BP/LR recombination reaction see the BLOCK-iT™ Pol II miR Validated miRNA Vector DuoPak manual for details.

To perform the standard BP and LR recombination reactions, see the Gateway® Technology with Clonase™ II manual for details.

Both manuals are available for downloading from www.invitrogen.com or by contacting Technical Service (see page 32).

Troubleshooting

Introduction

Review the information in this section to troubleshoot your experiments with the Validated miRNA Control Vector.

Transfection and RNAi Analysis

The table below lists some potential problems and possible solutions that may help you troubleshoot your transfection and knockdown experiments.

Problem	Reason	Solution
Low activity of the target gene observed	• Low transfection efficiency • Expression assay not performed correctly • Did not co-transfect the reporter plasmid expressing *lacZ* or luciferase	• See below • Be sure the expression assay was performed correctly. See page 18 for guidelines. • Be sure to co-transfect reporter plasmids for *lacZ* and luciferase knockdown experiments.
Low levels of gene knockdown observed due to low transfection efficiency	• Antibiotics added to the media during transfection if using Lipofectamine™ 2000 Reagent • Cells too sparse at the time of transfection • Not enough Validated miRNA Control plasmid DNA transfected • Not enough transfection reagent used	• Do not add antibiotics to the media during transfection. • Plate cells such that they will be 90-95% confluent at the time of transfection. • Increase the amount of Validated miRNA Control plasmid DNA transfected. • Optimize the transfection conditions for your cell line by varying the amount of transfection reagent used.
Low levels of gene knockdown observed (other causes)	Did not wait long enough after transfection before assaying for gene knockdown	Repeat the transfection and wait for a longer period of time after transfection before assaying for gene knockdown. Perform a time course of expression to determine the point at which the highest degree of gene knockdown occurs.
	The CMV promoter may be down-regulated in some cell lines (see page 10).	Use a different cell line or transfer the pre-miRNA cassette into appropriate destination vectors that allow tissue-specific or viral expression using Gateway® recombination reactions (see the BLOCK-iT™ Pol II miR Validated miRNA Vector manual available from www.invitrogen.com).
	Incorrect target site sequence present on the reporter plasmid	Confirm that the pre-miRNA target site (see page 2 for the sense target sequence) is present in your reporter plasmid prior to performing the knockdown experiments.

Continued on next page

Transfection and RNAi Analysis, continued

Problem	Reason	Solution
No gene knockdown observed when cells are transfected with the pcDNA™6.2-GW/EmGFP-miR-LMNA Validated miRNA construct	Used a cell line that does not express the human lamin A/C gene	Use a cell line that expresses the human lamin A/C gene (*e.g.* A549, HeLa, HEK 293, HT1080, COS-7).
	Used a cell line that expresses the lamin A/C gene, but does not share 100% homology with the pre-miRNA sequence	Use a human cell line that expresses the lamin A/C gene (*e.g.* A549, HeLa, HEK 293, HT1080) or use COS-7 cells. **Note:** The pcDNA™6.2-GW/EmGFP-miR-LMNA Validated miRNA construct expresses an miRNA targeted to the human lamin A/C gene. If you are using a non-human cell line, the lamin A/C gene may contain mismatches in the target region that renders the miRNA inactive.
Cytotoxic effects observed after transfection	Too much transfection reagent used	Optimize the transfection conditions for your cell line by varying the amount of transfection reagent used.
	Plasmid DNA not pure	Prepare purified plasmid DNA for transfection. We recommend using the PureLink™ Plasmid Purification Kits to prepare purified plasmid DNA.
No fluorescence signal detected with expression clone containing EmGFP	Incorrect filters used to detect fluorescence	Be sure to use the recommended filter sets for detection of fluorescence (see page 19). Be sure to use an inverted fluorescence microscope for analysis. If desired, allow the protein expression to continue for additional days before assaying for fluorescence. **Note:** We have observed reduced EmGFP expression from miRNA-containing vectors due to processing of the transcripts. In most cases, EmGFP expression should remain detectable.

Appendix

Recipes

Spectinomycin

Use this procedure to prepare a 10 mg/ml stock solution of spectinomycin.

1. Weigh out 50 mg of spectinomycin dihydrochloride (Sigma, Catalog no. S4014) and transfer to a sterile centrifuge tube.
2. Resuspend the spectinomycin in 5 ml of sterile, deionized water to produce a 10 mg/ml stock solution.
3. Filter-sterilize.
4. Store the stock solution at 4°C for up to 2 weeks. For long-term storage, store at -20°C.

LB (Luria-Bertani) Medium and Plates

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add antibiotic, if needed.
4. Store at room temperature or at 4°C.

For LB agar plates:

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, cool to ~55°C, add antibiotic if needed, and pour into 10 cm plates.
4. Let harden, then invert and store at 4°C.

Low Salt LB Plates with Blasticidin

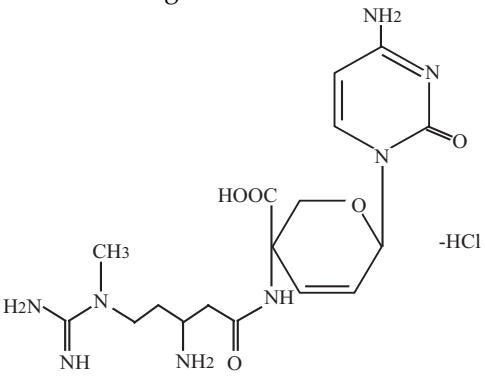
10 g Tryptone
5 g NaCl
5 g Yeast Extract

1. Combine the dry reagents above and add deionized, distilled water to 950 ml. Adjust pH to 7.0 with 1 N NaOH and bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle at 15 psi and 121°C for 20 minutes.
3. Allow the medium to cool to at least 55°C before adding the Blasticidin to 100 µg/ml final concentration.
4. Let harden, then invert and store at +4°C. Store plates at +4°C in the dark. Plates containing Blasticidin S HCl are stable for up to 2 weeks.

Blasticidin

Molecular Weight, Formula, and Structure

The formula for Blasticidin S is $C_{17}H_{26}N_8O_5$-HCl, and the molecular weight is 458.9. The diagram below shows the structure of Blasticidin.



Handling Blasticidin

Always wear gloves, mask, goggles, and protective clothing (*e.g.* a laboratory coat) when handling Blasticidin. Weigh out Blasticidin and prepare solutions in a hood.

Preparing and Storing Stock Solutions

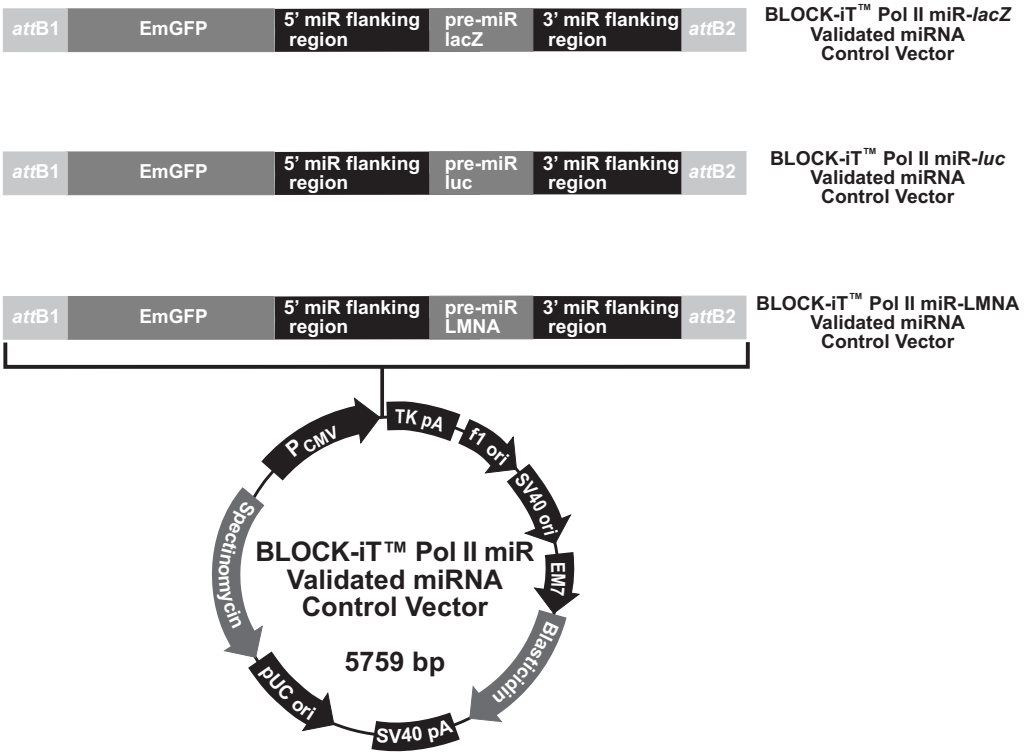
Blasticidin may be obtained separately from Invitrogen (see page v) in 50 mg aliquots. Blasticidin is soluble in water. Use sterile water to prepare stock solutions of 5 to 10 mg/ml.

- Dissolve Blasticidin in sterile water and filter-sterilize the solution.
- Aliquot solution in small volumes suitable for one time use (see next to last point below) and freeze at -20°C for long-term storage or store at +4°C for short-term storage.
- Aqueous stock solutions are stable for 1-2 weeks at +4°C and 6-8 weeks at -20°C.
- pH of the aqueous solution should be 7.0 to prevent inactivation of Blasticidin.
- Do not subject stock solutions to freeze/thaw cycles (**do not store in a frost-free freezer**).
- Upon thawing, use what you need and store the thawed stock solution at +4°C for up to 2 weeks.
- Medium containing Blasticidin may be stored at +4°C for up to 2 weeks.

Map and Features of pcDNA6.2™-GW/EmGFP-miR Validated miRNA Control Vector

Vector Map

The map below shows the elements of pcDNA6.2™-GW/EmGFP-miR Validated miRNA Control Vector. **The complete sequence for the vector is available from www.invitrogen.com or by contacting Technical Service (see page 32).**



Comments for BLOCK-iT™ Pol II miR Validated miRNA Control Vector
5759 nucleotides

CMV promoter: bases 1-588
*att*B1 site: bases 680-704
EmGFP: bases 713-1432
EmGFP forward sequencing primer site: bases 1409-1428
5' miR flanking region: bases 1492-1518
Pre-miRNA control insert: bases 1515-1582
3' miR flanking region: bases 1579-1623
*att*B2 site (C): bases 1652-1676
miRNA reverse sequencing primer site (C): bases 1667-1686
TK polyadenylation signal: bases 1705-1976
f1 origin: bases 2088-2516
SV40 early promoter and origin: bases 2543-2851
EM7 promoter: bases 2906-2972
Blasticidin resistance gene: bases 2973-3371
SV40 polyadenylation signal: bases 3529-3659
pUC origin (C): bases 3797-4470
Spectinomycin resistance gene (C): bases 4540-5550

(C) = Complementary strand

Continued on next page

Map and Features of pcDNA6.2™-GW/EmGFP-miR Validated miRNA Control Vector, Continued

Features of the Vector

The pcDNA6.2™-GW/EmGFP-miR Validated miRNA Control Vector (5759 bp) vector contains the following elements. All features have been functionally tested and the vector is fully sequenced.

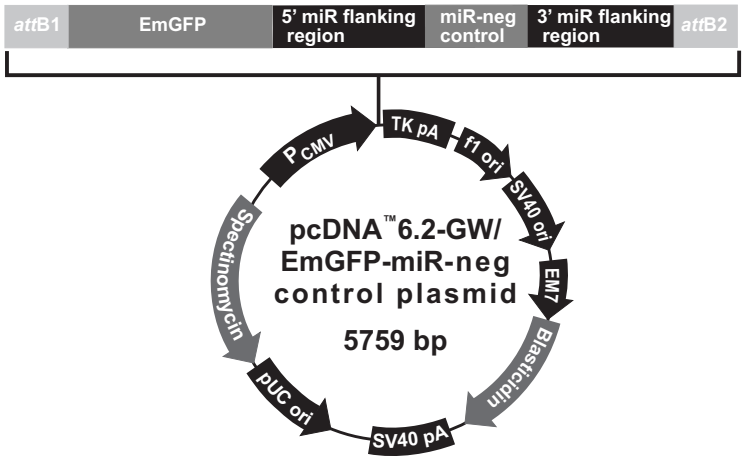
Feature	Benefit
CMV promoter	Permits high-level, constitutive expression of the gene of interest (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
*att*B1 and *att*B2 sites	Bacteriophage λ-derived recombination sequences that allow recombinational cloning of a gene of interest in the expression construct with a Gateway® destination vector (Landy, 1989).
EmGFP coding sequence	Allows visual detection of transfected mammalian cells using fluorescence microscopy.
EmGFP forward sequencing primer	Allows sequencing of the insert.
5′ miR flanking region	Allows formation of functional engineered pre-miRNA.
Pre-miR insert for *lacZ*, LMNA, and *luc*	Allows formation of a pre-miRNA hairpin sequence targeting *lacZ*, luciferase, or lamin A/C.
3′ miR flanking region	Allows formation of functional engineered pre-miRNA.
miRNA reverse sequencing primer	Allows sequencing of the insert.
TK polyadenylation signal	Allows transcription termination and polyadenylation of mRNA.
f1 origin	Allows rescue of single-stranded DNA.
SV40 early promoter and origin	Allows high-level expression of the selection marker and episomal replication in cells expressing the SV40 large T antigen.
EM7 promoter	Synthetic prokaryotic promoter for expression of the selection marker in *E. coli.*
Blasticidin (*bsd*) resistance gene	Permits selection of stably transfected mammalian cell lines (Kimura *et al.*, 1994).
SV40 polyadenylation signal	Allows transcription termination and polyadenylation of mRNA.
pUC origin	Permits high-copy replication and maintenance in *E. coli*.
Spectinomycin resistance gene (*aad*A1)	Allows selection of the plasmid in *E. coli* (Liebert *et al.*, 1999).
Spectinomycin promoter	Allows expression of the spectinomycin resistance gene in *E. coli*.

Map of pcDNA™6.2-GW/EmGFP-miR-neg Control Plasmid

pcDNA™6.2-GW/EmGFP-miR-neg Control Plasmid

The figure below shows the features of the pcDNA™6.2-GW/EmGFP-miR-neg Control Plasmid. The vector contains an insert between bases 1519 and 1578 that can form a hairpin structure just as a regular pre-miRNA, but is not predicted to target any known vertebrate gene allowing the use of the plasmid as a negative control for pre-miRNA experiments with pcDNA™6.2-GW/EmGFP-miR Validated expression vectors. The insert has been cloned according to the instructions in this manual.

The complete sequence of pcDNA™6.2-GW/EmGFP-miR-neg Control Plasmid is available for downloading from www.invitrogen.com or by contacting Technical Service (see page 32).



Comments for pcDNA™ 6.2-GW/EmGFP-miR-neg control plasmid 5759 nucleotides

CMV promoter: bases 1-588
*att*B1 site: bases 680-704
EmGFP: bases 713-1432
EmGFP forward sequencing primer site: bases 1409-1428
5' miR flanking region: bases 1492-1518
miR-neg control: bases 1519-1578
3' miR flanking region: bases 1579-1623
*att*B2 site (C): bases 1652-1676
miRNA reverse sequencing primer site (C): bases 1667-1686
TK polyadenylation signal: bases 1705-1976
f1 origin: bases 2088-2516
SV40 early promoter and origin: bases 2543-2851
EM7 promoter: bases 2906-2972
Blasticidin resistance gene: bases 2973-3371
SV40 polyadenylation signal: bases 3529-3659
pUC origin (C): bases 3797-4470
Spectinomycin resistance gene (C): bases 4540-5550
Spectinomycin promoter (C): bases 5551-5684

(C) = Complementary strand

Technical Service

Web Resources

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Continued on next page

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